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A novel 78-kDa fatty acyl-CoA synthetase (ACS1) of *Babesia bovis* stimulates memory CD4$^+$ T lymphocyte responses in *B. bovis*-immune cattle☆

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Abstract

Antigen-specific CD4$^+$ T lymphocyte responses contribute to protective immunity against *Babesia bovis*, however the antigens that induce these responses remain largely unknown. A proteomic approach was used to identify novel *B. bovis* antigens recognized by memory CD4$^+$ T cells from immune cattle. Fractions obtained from merozoites separated by continuous-flow electrophoresis (CFE) that contained proteins ranging from 20 to 83 kDa were previously shown to stimulate memory CD4$^+$ lymphocyte responses in *B. bovis*-immune cattle. Expression library screening with rabbit antiserum raised against an immunostimulatory CFE fraction identified a clone encoding a predicted 78 kDa protein. BLAST analysis revealed sequence identity of this *B. bovis* protein with *Plasmodium falciparum* fatty acyl coenzyme A synthetase (ACS) family members (PfACS1–PfACS11), and the protein was designated *B. bovis* acyl-CoA synthetase 1 (ACS1). Southern blot analysis indicated that *B. bovis* ACS1 is encoded by a single gene, although BLAST analysis of the preliminary *B. bovis* genome sequence identified two additional family members, ACS2 and ACS3. Peripheral blood lymphocytes and CD4$^+$ T cell lines from *B. bovis*-immune cattle proliferated significantly against recombinant ACS1 protein, consistent with its predicted involvement in protective immunity. However, immune sera from cattle recovered from *B. bovis* infection did not react with ACS1, indicating that epitopes may be conformationally dependent.

Keywords: *Babesia bovis*; Fatty acyl-CoA synthetase; ACS; T lymphocytes

1. Introduction

Babesial parasites infect many mammalian species, notably causing disease in humans, dogs, horses, and cattle [1,2]. In cattle, *Babesia bovis* infection results in a virulent systemic disease similar to *Plasmodium falciparum* malaria in humans [3]. Both parasites can sequester in organ microcapillary beds, resulting in cerebral dysfunction, kidney failure, and pulmonary edema in addition to anemia [3]. Cattle that recover from *B. bovis* infection, either naturally, or following prophylactic treatment, remain persistently infected yet resistant to clinical disease following reinfection with the homologous strain.

The most efficacious vaccines for bovine babesiosis are live organisms attenuated following passage in splenectomized

Abbreviations: ACS, fatty acyl-CoA synthetase; CFE, continuous-flow electrophoresis; CM, cell membrane; HSS, high speed supernatant; MBP, maltose binding protein; MSA-1, merozoite surface antigen-1; MSP5, *Anaplasma marginale* major surface protein-5; P0, ribosomal phosphoprotein P0; RAP-1, rhoptry associated protein-1; URBC, uninfected red blood cells

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calves, and to date no subunit vaccines have provided better immunity. However, non-living vaccines consisting of killed parasites, parasite extracts, or antigen fractions have induced some protection (defined as reduced parasitemias and diminished reduction in erythrocyte packed cell volume) in cattle challenged with *B. bovis* [4–6]. The serologically immunodominant *B. bovis* merozoite surface antigen-1 (MSA-1), did not afford protection against challenge even though MSA-1-specific immune serum blocked merozoite invasion in vitro [7–9]. The most protective antigens that have been described are secreted proteins, and these are neither abundant nor serologically dominant [6,10–19]. However, a recent vaccine trial with a recombinant version of one of these proteins, *B. bovis* rhoptry associated protein-1 (RAP-1), which also associates with the merozoite surface, did not provide any measurable protection in spite of induction of potent cell mediated and antibody responses predicted to be required for protective immunity [20]. Thus, it may be that a non-living vaccine will need to incorporate multiple immunogenic proteins or their epitopes. Furthermore, to provide protection to a large number of animals in a herd situation, candidate vaccine antigens should express epitopes conserved among parasite strains and recognized by animals expressing a broad repertoire of MHC class II molecules.

We have used a proteomic approach to identify novel immunogenic *B. bovis* proteins that does not depend on merozoite surface localization or serological immunodominance. We hypothesized that this approach could reveal previously unrecognized subdominant antigens. Antigens were selected by their ability to elicit memory $CD4^+$ T lymphocyte proliferation in genetically diverse cattle that had recovered from *B. bovis* infection and were protected against homologous strain challenge [4]. Using continuous-flow electrophoresis (CFE) to separate parasite proteins by size, several protein fractions were identified that stimulated strong proliferation of *B. bovis*-specific $CD4^+$ T cell clones and lines [21,22]. *B. bovis* expression library immunoscreening was performed using antisera produced against selected CFE fractions. The present study reports the cloning and expression of a novel 78 kDa protein, fatty acyl-CoA synthetase 1 (ACS1) of *B. bovis*. Recombinant ACS1 elicited memory $CD4^+$ T lymphocyte responses in *B. bovis*-immune cattle. BLAST analysis of *B. bovis* genomic sequences identified two related proteins, designated ACS2 and ACS3. All three *B. bovis* acyl-CoA synthetase (ACS) proteins share sequence identity with *P. falciparum* and *Theileria* spp. family members and contain two highly conserved motifs involved in binding fatty acids and ATP.

2. Materials and methods

2.1. B. bovis-immune cattle

Cow C97 (Brahman × Angus) was infected with the Mexico strain of *B. bovis*, and Holstein steer 01B49 was infected with the Texas T_2Bo strain of *B. bovis*. Both animals became parasitemic and were treated with 3.0 mg of berenil (diaminazine acetu-rate: Sigma)/kg of body weight as described in detail elsewhere [20,23]. Animal C97 was immune to homologous strain challenge [23], and animal 01B49 was not challenged. Holstein steer calf 00B16 was infected with frozen stabilate of the MO7 clone of *B. bovis* (Mexico strain) and developed peak temperature (105.6 °F) on day 6 and peak reduction in packed cell volume on day 9 post-infection. The calf did not require treatment as parasites were not observed on blood smears, although parasites were observed on smears from other calves inoculated with the same stabilate at the same time. Angus × Simmental steer calf 00B17 was infected with frozen *B. bovis* (Mexico strain) stabilate, developed peak parasitemia (0.5%) and peak temperature (105.3 °F) on day 10 post-infection, resolved the infection without treatment, and was immune to challenge one month later. Cattle became serologically positive for *B. bovis* ([20,23] and unpublished observations). The MHC class II *DRB3* alleles were defined by PCR-RFLP analysis of exon 2 [24] and analysis was kindly performed by Dr. Harris Lewin, Department of Animal Science, University of Illinois. The nomenclature of the alleles is described on the bovine leukocyte antigen (BoLA) nomenclature websites (http://www.projects.roslin.ac.uk/bola and http://www.ebi.ac.uk/ipd/mhc/bola). The *DRB3* RFLP types are for cow C97:15/34; for steer B49:11/27; for steer 00B16:16/22; for steer 00B17:18/7.

2.2. Babesial parasite strains and antigen preparation

In vitro microaerophilous cultures of *B. bovis* were maintained in bovine erythrocytes obtained from uninfected cattle [23]. Antigens were prepared by homogenization of culture-derived merozoites or uninfected erythrocytes (URBC) with a French pressure cell (SLM Instruments) and ultracentrifugation as described [23]. Briefly, merozoites were harvested from culture following CO_2 deprivation achieved by 18 h incubation in medical grade oxygen at 37 °C in serum-free HL-1 medium (Ventrex Laboratories), pelleted by a series of 10 min centrifugations at 140, 140, 180, and 220 × *g*, with a final 15 min centrifugation at 5000 × *g* and resuspended in PBS containing 25 μg/ml protease inhibitors E-64 and Antipain (Boehringer Mannheim). The merozoites were disrupted by two passages through a French pressure cell (SLM Instruments) under a chamber pressure of 1500 lb/in.^{2}, and the homogenate was centrifuged for 1 h at 145,000 × *g*. The soluble high speed supernatant (HSS) and membrane and organelle-enriched pellet (CM) were collected and CM was resuspended in PBS. Antigens were stored at −70 °C.

2.3. cDNA library construction and screening

Purified *B. bovis* (Mexico strain) merozoite poly(A) RNA was used to construct a directional library using a ZAP Express cDNA Synthesis Kit (Stratagene) as described [25]. The *B. bovis* library was screened as described [25] with a rabbit antiserum (5606) raised against a 20 kDa fraction of whole *B. bovis* proteins fractionated by CFE [21]. Clone *pbbo26* encoded a predicted 60 kDa protein. BLAST analysis of the NCBI database revealed a significant similarity with the C-region of

P. falciparum fatty acyl-CoA synthetase family, suggesting that clone *pbbo26* was truncated at the 5′ end. To obtain the full-length sequence of *pbbo26*, total RNA was extracted using the TRIzol reagent (Invitrogen) from *B. bovis* merozoites. The first-stranded DNA was synthesized from 3 μg of the total RNA with oligo(dT) and precipitated with spermine. The cDNA was resuspended with 68 μl of H_2O, and a poly(dG) was introduced in a 100-μl reaction mixture containing 1 mM dGTP (Perkin-Elmer Cetus), 30 units of deoxynucleotidyl-transferase (TdT) (Invitrogen), and 5× TdT buffer (Invitrogen) and incubated for 1 h at 37 °C. The 5′ end of *pbbo26* was amplified by PCR using a mixture of the ANpolyC primer (5′-GCATGCGCGCGGCCGCGGAGGCCCCCCCCCCCCCC-3′) and the AN primer (5′-GCATGCGCGCGGCCGCGGA-GGCC-3′) at a ratio of 1:9 as a forward primer and a *pbbo26*-specific sequence 5′-AGTGGCAATGGCATTGCGGT-3′ as a reverse primer. The PCR parameters were 94 °C for 10 min followed by 5 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min and 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 2 min, and a final extension of 72 °C for 7 min. The amplicon was ligated into pCR2.1-TOPO TA cloning vector (Invitrogen) and sequenced. After completion of the 5′ end sequence of *pbbo26* cDNA, one primer set (forward: 5′-ATGTCGGCAG-AATTGTTTGG-3′; reverse: 5′-GTGGCCGTACATTGCGTT-GA-3′) was used to amplify the complete *pbbo26* mRNA sequence using RT-PCR. The PCR product was cloned into pCR2.1 vector and sequenced (GenBank accession number AF331454). Because the amino acid sequence predicted from the full-length *pbbo26* indicated that *pbbo26* was an orthologue of *P. falciparum* ACS family members, *pbbo26* was designated *B. bovis* fatty acyl-CoA synthetase 1.

2.4. Sequence analysis of B. bovis genomic DNA

B. bovis (T_2Bo strain) bacterial artificial chromosome (BAC) library nylon filters (AMPLICON Express, Pullman, WA, USA) were screened with full-length digoxigenin (DIG)-labeled *BboACS1* amplified using the PCR DIG Probe Synthesis Kit (Roche). The filters were pre-hybridized with DIG Easy Hyb buffer (Roche) at 42 °C for 2 h and hybridized with DIG Easy Hyb buffer containing the DIG-*BboACS1* probe at 42 °C overnight. After hybridization, the filters were washed three times at 68 °C in washing buffer containing 0.1× SSC and 0.1% SDS. The filters were treated with anti-DIG-AP conjugate (Roche) and the luminescence signal was detected by exposure to Hyperfilm (Amersham). The 9D2 BAC clone was confirmed to contain the entire *BboACS1* gene by PCR using the primer set described above. Since there was no *Bam*HI site in the *BboACS1* gene, the 9D2 BAC clone DNA was digested with *Bam*HI and ligated into the *Bam*HI site of the pBluescript II (Stratagene) multiple cloning sites using T4 DNA ligase (GIBCO BRL), and introduced into TOP10 competent cells (Invitrogen). Colonies were screened with the DIG-labeled *BboACS1* probe using colony hybridization. A clone containing a 4.8 kb *Bam*HI fragment was detected as a positive colony and the entire fragment was subsequently sequenced (GenBank accession number AY534753).

2.5. Southern hybridization analysis

Southern hybridization was performed according to the DIG System Users Guide (Boehringer Mannheim). Briefly, *B. bovis* Mexico strain genomic DNA was digested with *Eco*RI, *Bam*HI, *Eco*RV, *Pst*I, *Hin*dIII, *Xho*I, *Sal*I, *Nde*I, *Bgl*II, and *Xba*I as recommended by the manufacturer (GIBCO BRL). Digested DNA (0.6 μg per lane) was separated by agarose gel electrophoresis, transferred to a nylon membrane (Boehringer Mannheim) and fixed by UV cross-linking. Pre-hybridization was performed as described above and the membrane was hybridized at 42 °C overnight with the DIG-labeled *BboACS1* probe in Easy Hyb buffer. After hybridization, the membrane was washed three times at 68 °C in washing buffer containing 0.1× SSC and 0.1% SDS. Detection of probe–target hybrids was performed using anti-DIG-AP conjugate (Roche) and the luminescence signal was detected by exposure to Hyperfilm (Amersham).

2.6. Cloning of BboACS1 homologues

Because Southern blotting analysis did not reveal evidence of *BboACS1*-homologous genes, the full-length amino acid sequence of *B. bovis* ACS1 (GenBank accession number AAS37667) was used to search the entire T_2Bo strain *B. bovis* genomic sequences provided by the Institute for Genome Research (TIGR) using TBLASTN. The *B. bovis* genome sequence is accessible for BLAST analysis at the following URL: http://www.vetmed.wsu.edu/research_vmp/babesia-bovis/index.asp. In addition to the expected *BboACS1* sequence, two sequences were identified and designated as *BboACS2* (GenBank accession number AY898655) and *BboACS3* (GenBank accession number AY898656). To obtain the complete *BboACS2* and *BboACS3* mRNA sequences from the Mexico strain of *B. bovis*, RNA transcript rapid amplification of cDNA ends (RACE) was performed using the SMART RACE Kit (BD Biosciences, Clontech, Palo Alto, CA, USA). For *BboACS2*, a primer 5′-CGTATTTATCATGGCGGGTGTCGCT-3′ was used as a 5′ RACE reverse primer. For *BboACS3*, 5′-CAGTGCAAGGTGATCAGGTTCAGCTG-3′ was used as a 5′ RACE reverse primer. The PCR was carried out according to the manufacturer's instructions. Each PCR product was subsequently cloned into pCR2.1 vector and sequenced. For the 3′ RACE, forward primers were designed based on the sequences obtained by the 5′ RACE. The primers were 5′-CGATATATTTAAAGGCCTGGGTCAAACAACA-3′ and 5′-TGATGTGGCATTACATGGCCATATCTTC-3′ for *BboACS2* and *BboACS3*, respectively. Each 3′ RACE PCR product was cloned into pCR2.1 vector and sequenced. We confirmed that these 3′ RACE products, *BboACS2* (2.3 kb) and *BboACS3* (2.4 kb), each contained the full-length open reading frame. The GenBank accession numbers for *BboACS2* and *BboACS3* mRNAs are AY898653 and AY898654, respectively.

2.7. Phylogenetic analysis

A phylogenetic analysis of predicted amino acid sequences obtained from *B. bovis* ACS1–ACS3 (GenBank accession

numbers AAS37667, AY898653, and AY898654), *P. falciparum* ACS1–ACS3 and ACS5–ACS11 (AD53966, CAB39122, AAN36596, CAD51056, CAD49109, AAN36096, AAC71928, AAC71926, CAD51614, and AAN36462), *Theileria parva* long-chain fatty acid CoA ligases designated TpFACLa-c (EAN32393, EAN33363, and EAN33757), *Theileria annulata* long-chain acyl CoA synthetase (TaACS; CAI74871), *T. annulata* long-chain fatty acid CoA ligase (TaFACL; CAI73914), and *T. annulata* TaFCL5 (CAI73571) was performed. The multiple sequence alignment was generated using MegAlign (DNASTAR). The trees were generated using PAUP (Phylogenetic Analysis Using Parsimony 4.0 beta 10 (Altivec)). Related sequences were aligned using MegAlign (DNASTAR). Alignment output was used to construct a distance phylogram using the neighbor joining algorithm with bootstrapping, and the related *Bos taurus* long-chain fatty acid transport protein 1 (GenBank accession number Q3ZKN0) was used as an outgroup.

2.8. Expression of BboACS1

Truncated recombinant ACS1 (t-ACS1) protein was prepared as a maltose binding protein (MBP) fusion protein. Briefly, plasmid *pbbo26* encoding aa 188–702 of ACS1 was amplified from *B. bovis* (Mexico strain) DNA by PCR with the GeneAmp Kit (Perkin-Elmer) using forward and reverse primer sequences located at the 5′ and 3′ ends of the *pbbo26* and modified to include an *Eco*RI restriction site at both ends. The PCR products were digested with *Eco*RI and subcloned into the pMAL2c vector (New England Biolabs). *Escherichia coli* XL-1 Blue (Stratagene) containing the recombinant plasmids encoding the maltose binding protein or MBP fused to t-ACS1 were cultured and protein was induced with IPTG, and purified by affinity chromatography on amylose resin columns (New England Biolabs).

To generate a full-length ACS1 his-tagged protein, the full-length *BboACS1* was amplified by RT-PCR and subcloned into the pBAD/Thio-TOPO expression vector (Invitrogen) as recommended by the manufacturer. Primers were identical to those used for designing the DIG-*pbbo26* probe for Southern blotting. These constructs were introduced into *E. coli* TOP-10 competent cells. The direction and frame were confirmed by sequencing the complete insert and flanking regions. For the expression of each recombinant antigen, a single recombinant *E. coli* colony was inoculated in 2 ml of Luria broth (LB) containing 50 μg of ampicillin per milliliter and incubated overnight at 37 °C with shaking. The culture was inoculated into 150 ml of LB and incubated for 2 h at 37 °C with shaking. Protein expression was induced with L-(+)-arabinose (0.2% final concentration) for 4 h. The recombinant protein was purified by affinity to a Ni^{2+} column using ProBond™ resin (Invitrogen) as detailed elsewhere [18]. Proteins were analyzed by SDS-PAGE and Coomassie blue staining. For use in T cell proliferation assays, proteins were dialyzed extensively against PBS and quantified using the Micro BCA Protein Assay Reagent Kit (Pierce).

2.9. Antibody production and immunoblotting

Six mice were immunized with 20 μg per inoculation of recombinant his-tagged ACS1 in complete Freund's adjuvant and boosted three times in incomplete Freund's adjuvant. After confirming seroconversion by immunoblotting described below, the sera were pooled 7 days after the last boost. Sera were extensively adsorbed with purified recombinant *Anaplasma marginale* major surface protein-5 (MSP5) expressed in the same vector [20] and stored at −20 °C.

Immunoblotting was performed essentially as described [26] using mouse anti-ACS1 sera or mouse antiserum directed against another *B. bovis* protein, phosphoribosomal protein P0 (Norimine and Brown, unpublished observations; GenBank accession number AF498365), and pre- or post-*B. bovis* infection bovine sera absorbed with *E. coli* lysate. Antigens loaded were 0.2 μg per lane recombinant ACS or P0 protein, both expressed in pBAD/Thio-TOPO, and 10 μg per lane *B. bovis* CM or HSS derived from the Mexico strain. Antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse, or goat anti-bovine IgG (Kirkegaard and Perry) and Enhanced Chemiluminescence (Amersham), or with alkaline phosphatase-conjugated goat anti-rabbit IgG (Kirkegaard and Perry) and NBT/BCIP substrate (Sigma).

2.10. Lymphocyte proliferation assays

PBMC from calves 00B16 and 00B17 were obtained prior to infection and at 3–9 weeks post-*B. bovis* infection and were tested for proliferation to recombinant t-ACS1-MBP. Positive control recombinant protein was *B. bovis* rhoptry associated protein-1-MBP [17] and negative control antigens were recombinant MBP alone expressed in the pMAL vector and *A. marginale* MSP5. Additional antigens included CM or HSS prepared from the Mexico strain of *B. bovis* and uninfected erythrocyte membranes as a negative control antigen. Briefly, 2×10^5 PBMC were cultured in 96-well plates with 1–25 μg/ml of antigen for 6 days, radiolabeled with 0.25 μCi [^{3}H]thymidine (Dupont New England Nuclear) for 6–18 h, then harvested and counted in a scintillation counter. In addition, $CD4^+$ T cell lines were generated from *B. bovis*-immune animals C97 (infected with *B. bovis* for 12 years) and B49 (infected with *B. bovis* for 1 year). Briefly, PBMC were cultured for 1 week in 24-well plates with 5 μg *B. bovis* CM antigen per milliliter of complete RPMI-1640 medium [23], and then either restimulated with antigen and fresh autologous, irradiated PBMC as a source of antigen presenting cells (APC) on a weekly basis for up to 8 weeks, or cultured with APC without antigen every other week. $CD8^+$ T cells and γδ T cells were depleted from B49 lymphocytes after 2 weeks of culture (1 week with antigen and 1 week without) using monoclonal antibodies and complement mediated lysis as described [26] prior to testing. Antibody-based depletion of γδ and $CD8^+$ cells yielded a population of lymphocytes that were >95% $CD4^+$. Cells were washed and assayed for antigen-specific proliferation for 3 days in 96-well plates using 3×10^4 cells per well with 5×10^5 APC and antigen. Proliferation was measured by [^{3}H]thymidine incorporation and results are presented as the

mean cpm ± 1S.D. of triplicate cultures and as the stimulation index, determined as the mean cpm of cells cultured with antigen/mean cpm of cells cultured with medium. The Student's one-tailed *t*-test was used to determine significant differences in stimulation of lymphocyte lines by parasite antigen compared with no antigen or control URBC, MSP5, or MBP antigen.

3. Results

3.1. Cloning and sequence analysis of ACS1

In previous studies, we described *B. bovis* merozoite antigens separated by CFE, including fractions of 78–82 kDa, that stimulated memory T lymphocyte responses from several *B. bovis*-immune cattle [21,22]. To identify immunostimulatory proteins, rabbit antisera were produced against antigen fractions with apparent molecular weights of 20, 40, 51/52, and 60 kDa proteins that stimulated memory $CD4^+$ T lymphocytes [21], and were used to screen a *B. bovis* cDNA expression library. The antiserum against the 20 kDa fraction identified *B. bovis* Hsp20 [26] as well as higher molecular weight proteins including Hsp70 and Hsp90 [25], indicating that this fraction contained both intact and degraded proteins. In addition, this serum recognized clone *pbbo26* that had sequence identity to the 3′ region of *P. falciparum* ACS1, indicating that the *B. bovis* clone was apparently truncated at the 5′ end. Complete sequence of the *B. bovis* ACS homologue obtained by 5′ RACE predicted a full-length 687 amino acid protein with an estimated molecular weight of 78 kDa (Fig. 1). This *B. bovis* ACS homologue was designated ACS1. Comparison of *B. bovis* ACS1 with the ACS orthologues from *P. falciparum* (PfACS1–PfACS11) [27] showed the highest degree of similarity with PfACS11, which has 32% amino acid sequence identity (Fig. 1A and data not shown).

3.2. B. bovis ACS family members

B. bovis Mexico strain genomic DNA was digested with restriction enzymes that generate no cuts (*Bam*HI, *Eco*RI, *Xho*I, *Sal*I, *Nde*I, and *Bgl*II), one cut (*Eco*RV and *Xba*I), or two cuts (*Pst*I and *Hin*dIII) within the *BboACS1*-coding region. Southern blotting generated internal fragments of the predicted sizes when DNA was digested with *Pst*I or *Hin*dIII, which were 660 and 882 bp, respectively (data not shown). The number of bands generated with all enzymes indicated that *BboACS1* is a single copy gene. Lowering hybridization stringency also failed to detect the homologous genes (data not shown). The *Bam*HI fragment obtained from the 9D2 BAC clone was 4.8 kb, although Southern blotting analysis of the Mexico strain DNA revealed a fragment slightly smaller than 4.4 kb. This discrepancy indicates sequence variation in *B. bovis* genomic DNA from the Mexico strain for the Southern blotting and the T_2Bo strain used for constructing the BAC clone.

Because Southern blotting analysis did not reveal evidence of *BboACS1*-homologous genes, the full amino acid sequence of ACS1 was used to search the preliminary *B. bovis* genome sequences of the T_2Bo strain using TBLASTN. *BboACS1* was identified and was 99.6% identical in sequence to that of the Mexico strain, with 3/681 amino acid differences (data not shown). Two long related sequences were also identified and designated as *BboACS2* and *BboACS3*. The predicted amino acid sequences encoded by *B. bovis* Mexico strain mRNA, obtained following RACE, indicate homology with ACS1, ranging from 30 to 43% amino acid identity (Fig. 1A). *BboACS2* encodes a protein of 670 amino acids with a predicted molecular weight of 75 kDa, and *BboACS3* encodes a protein of 681 amino acids with a predicted molecular weight of 77 kDa. These predicted amino acid sequences were identical in ACS2 and 99.0% identical in ACS3 (7/681 amino acid differences) when *B. bovis* Mexico and T_2Bo strains were compared (data not shown). ACS1–ACS3 have an ATP binding motif in the central region that is conserved among members of the superfamily of the adenylate forming enzymes [28]. This is the [S/T/G]-[S/T/G]-G-[S/T]-[T/S/E]-[G/S]-X-[P/A/L/I/V/M]-K motif that is also conserved in PfACS family members [29] and identified in *B. bovis* ACS1 as TSGTSGMPKG at residues 265–274, in ACS2 as TSGTSGIPKG at residues 241–250, and in ACS3 as TSGTSGVPKG at residues 250–259 (Fig. 1A). In addition, the C-terminal region of the ACS family members is highly conserved between apicomplexan protozoa including *P. falciparum*, *T. parva*, *T. annulata*, and *B. bovis* (Fig. 1A). This region contains the ACS signature motif that plays a critical role in fatty acid binding [27,29]. In *B. bovis*, this motif was identified in ACS1 as EGWYHTGDVVELLPSMGVKILDRAR at residues 524–548, in ACS2 as DGWLLTGDIAELLPNGAIKIIDRRK at residues 503–527, and in ACS3 as DGWLHTGDVVEIQPSGAIRIIDRVK at residues 511–535 (Fig. 1A).

The amino acid sequences of *B. bovis*, *P. falciparum*, *T. parva*, and *T. annulata* ACS family members were aligned and used to construct a distance phylogram using a neighbor joining algorithm with bootstrapping and the related long-chain fatty acid transport protein of *B. taurus* as an outgroup (Fig. 1B). Results of this analysis suggests that these sequences fall into two distinct clades, with *B. bovis* ACS1–ACS3 forming a clade with *P. falciparum* ACS10 and ACS11 and the *Theileria* proteins, and the remaining *P. falciparum* ACS proteins forming a separate clade.

3.3. Localization of ACS1 in merozoites and antigenicity for cattle

Immunoblotting was performed using monospecific mouse antiserum raised against either recombinant *B. bovis* ACS1 or control P0 protein. Antibodies were reacted with CM or HSS antigens prepared from the Mexico strain of *B. bovis*. As seen in Fig. 2, ACS1, which migrates at approximately 78 kDa, is detected in the membrane and organelle-enriched CM of *B. bovis*. Upon longer exposure, a faint 78 kDa band also appeared in the HSS (data not shown), suggesting that this protein mainly associates with membranes or organelles, but may be weakly present in the cytosolic merozoite fraction. However, the results do not rule out a possible artifactual dissociation of the protein from a membrane component. The antiserum also recognized a ∼94 kDa band in the recombinant ACS protein that was absent

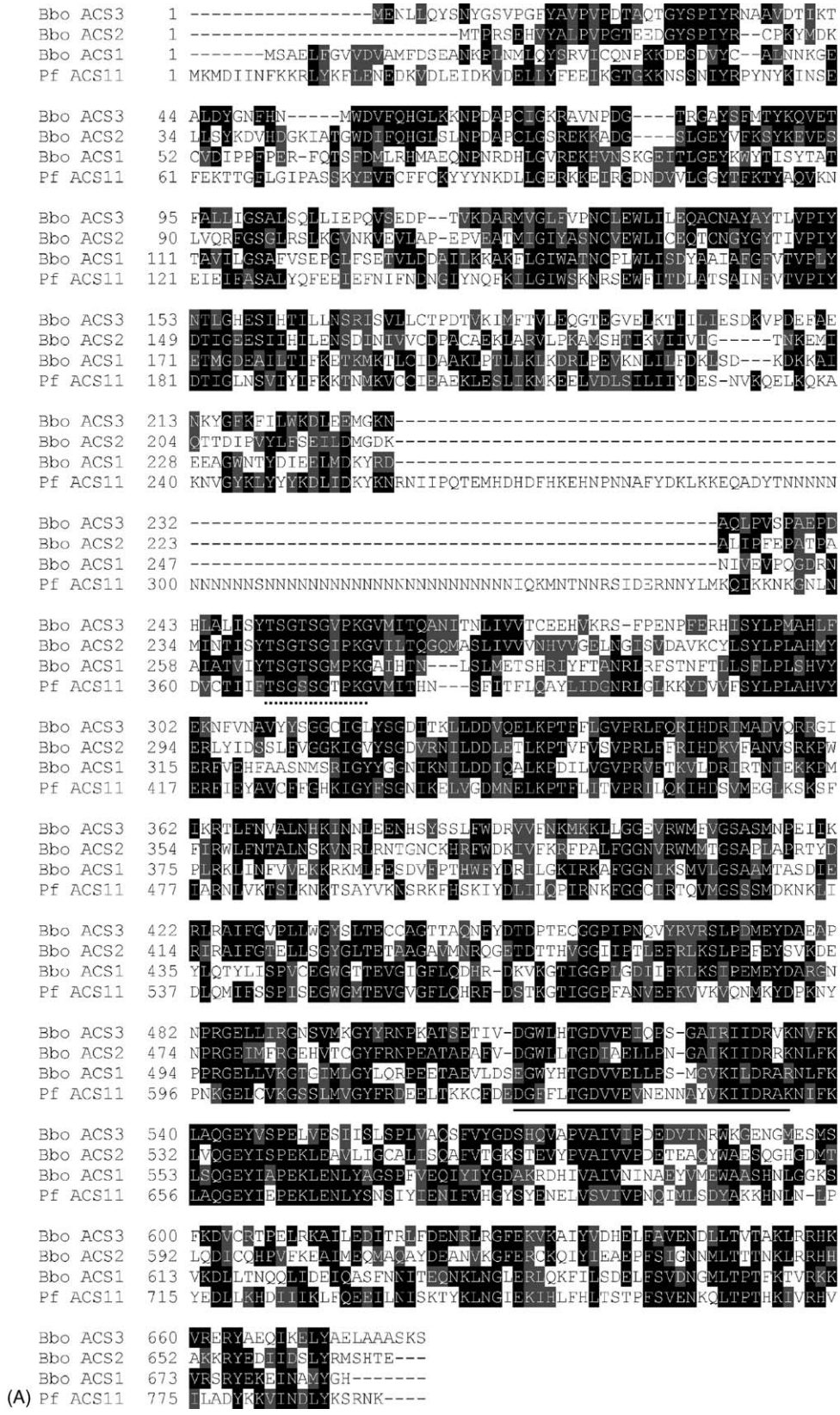

Fig. 1. Predicted amino acid sequence alignment and phylogenetic analysis of *B. bovis*, *P. falciparum*, and *Theilera* ACS1 proteins. Predicted amino acid sequences of full-length *B. bovis*, ACS1–ACS3 and *P. falciparum* ACS11 are shown in panel A. The [S/T/G]-[S/T/G]-G-[S/T]-[T/S/E]-[G/S]-X-[P/A/L/I/V/M]-K motif is indicated by a dashed line and the fatty acid binding domain signature motif is indicated by a solid line. Areas of amino acid identity have a black background, and grey shading indicates conservative amino acid substitutions. A phylogram showing distance and bootstrap values represented as percentage is shown in panel B. The bar represents 0.1 substitutions per site.

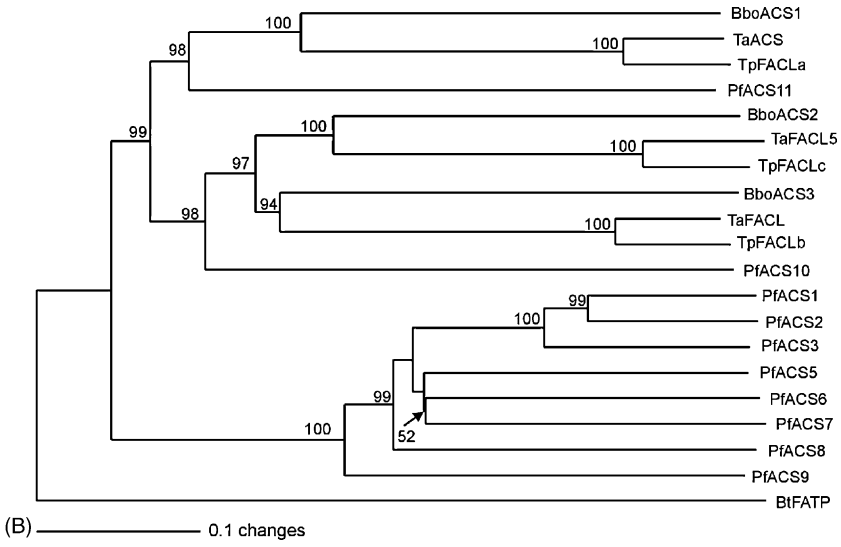

Fig. 1. (*Continued*).

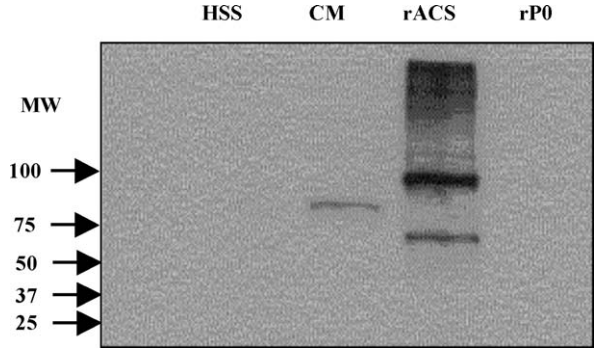

Fig. 2. Localization of ACS in the membrane and organelle-enriched fraction of *B. bovis*. CM and HSS antigens prepared from *B. bovis* (Mexico strain) (10 μg per lane) or recombinant *B. bovis* ACS1 and P0 proteins (0.2 μg per lane) were subjected to SDS-PAGE and immunoblotting with a 1:200 dilution pooled mouse anti-ACS peptide-specific antisera.

in the control recombinant P0 protein constructed with the same vector. The larger molecular weight of the recombinant protein results from the additional sequence of ∼16 kDa contributed by the thioredoxin fusion protein. The smaller ∼60 kDa protein band visible in the recombinant ACS1 may be the result of protein degradation.

To determine whether ACS1 is antigenic for cattle infected with *B. bovis*, immune sera from cattle C97 and B49, previously shown to recognize other *B. bovis* proteins including RAP-1 [20,23], were tested by immunoblotting with recombinant ACS1. However, neither of these antisera recognized this protein (data not shown). Sera were also tested from additional *B. bovis* infected cattle, and these were also negative by immunoblotting (data not shown). In contrast, the rabbit antiserum raised against a native protein fraction that was used to originally isolate clone *pbbo26* by expression library screening, did react with recombinant ACS1 on immunoblots (data

Table 1
PBMC from *B. bovis*-immune cattle proliferate in response to truncated ACS1

Antigen	Mean cpm incorporated by PBMC (stimulation index)[a]			
	Animal 00B16		Animal 00B17	
	Pre-infection	Post-infection	Pre-infection	Post-infection
URBC	278 ± 78	573 ± 260	919 ± 657	913 ± 585
B. bovis CM	519 ± 235 (1.9)	**8713 ± 1112 (15.2)**[b]	868 ± 2 (0.9)	**5599 ± 1094 (6.1)**
B. bovis HSS	213 ± 33 (0.8)	**9239 ± 2837 (16.1)**	1384 ± 279 (1.5)	**8363 ± 1935 (9.2)**
MBP	ND[c]	2139 ± 728	ND	1686 ± 667
t-ACSl-MBP	394 ± 18 (1.4)	**10736 ± 2758 (5.0)**	1630 ± 750 (1.8)	**15133 ± 5915 (9.0)**
RAP-1-MBP	693 ± 14 (2.5)	**31139 ± 8905 (14.6)**	2011 ± 665 (2.2)	**13857 ± 1366 (8.2)**

[a] Cell lines were propagated by stimulation with *B. bovis* CM for 2–8 weeks and 3×10^4 lymphocytes were washed and cultured with 2×10^5 APC and antigen for 3 or 4 days. Results are presented as the mean cpm ± 1S.D. of triplicate cultures with 5 μg/ml (calf 00B16) or 25 μg/ml (calf 00B17) URBC and *B. bovis* antigens or 5 μg/ml MBP and *BboACS1*-MBP or RAP-1-MBP and as the stimulation index in parentheses.

[b] Responses were considered to be significant ($P < 0.05$) if the stimulation index is >3.0 and the mean cpm > 1000, and are indicated in bold-faced type when *B. bovis* antigens were compared with URBC and recombinant *B. bovis*-MBP fusion proteins were compared with URBC (lymphocytes obtained pre-infection) or MBP (lymphocytes obtained post-infection).

[c] Not determined.

not shown). Thus, in the native state, ACS1 does not appear to be serologically immunodominant and does not stimulate an antibody response detectable by immunoblotting during *B. bovis* infection. It is possible that in cattle, antibody responses to ACS1 are directed solely to conformationally dependent epitopes.

3.4. $CD4^+$ T lymphocytes from *B. bovis*-immune cattle proliferate in response to ACS1

Previous studies with CFE-fractionated merozoites identified proteins ranging from 62 to 83 kDa that stimulated proliferation of $CD4^+$ T cell lines from several immune animals, including animal C97 used in the present study [21,22]. To determine whether ACS1 is recognized by T lymphocytes from *B. bovis*-immune cattle, PBMC from calves 00B16 and 00B17, that had recently been infected with *B. bovis*, or short-term T cell lines from cattle C97 and B49, infected either more than 10 years or approximately 1 year earlier, respectively, were tested for ACS-specific proliferation. Significant proliferation was observed by PBMC from recently infected animals obtained 3–5 weeks post-challenge, but not by PBMC obtained before challenge, when stimulated with t-ACS1-MBP or RAP-1-MBP as compared with MBP alone (Table 1). Significant proliferation against *B. bovis* and ACS1 was also manifested by T lymphocyte lines from animal C97 and from $CD4^+$ T cell lines obtained from more recently infected animal B49 (Table 2). Truncated t-ACS1-MBP or the full-length ACS1 expressed as a his-tagged fusion protein stimulated similar levels of proliferation, whereas control MBP or *A. marginale* MSP5, expressed as a his-tagged protein in the same vector as ACS1, did not elicit proliferation. These data are representative of two experiments performed with cell lines obtained at different times from these immune animals.

Table 2
$CD4^+$ T lymphocyte lines from *B. bovis*-immune cattle respond to full-length ACS1

Antigen	Mean cpm incorporated by T lymphocyte lines[a]	
	Animal C97	Animal B49
URBC	223 ± 33	950 ± 225
B. bovis CM	**120939 ± 4845**[b]	**35178 ± 1640**
MSP5	528 ± 62	2307 ± 558
ACS1	**6971 ± 1981**	**7502 ± 21**
RAP-1	ND	**13494 ± 4546**
MBP	702 ± 76	ND[c]
t-ACSl-MBP	**5614 ± 1301**	ND
RAP-1-MBP	**17212 ± 856**	ND

[a] T cells (3×10^4) were cultured with 2×10^5 APC and antigen for 3 days, radiolabeled, harvested, and counted. Results are presented as the mean cpm ± 1S.D. of triplicate cultures with 25 μg/ml (cow C97) or 10 μg/ml (cow B49) antigen.

[b] Statistically significant differences in proliferation ($P < 0.01$) were observed when ACS1 was compared with MSP5, t-ACSl-MBP, or RAP-1-MBP were compared with MBP, and *B. bovis* was compared with URBC by the one-tailed Student's *t*-test.

[c] Not determined.

4. Discussion

The proteomic approach that we have taken to select potential vaccine antigens of babesial parasites used $CD4^+$ T cells as probes to identify antigens that elicit memory responses from cattle immune to reinfection. $CD4^+$ T cells are critical for inducing and maintaining both humoral and cell-mediated immunity against babesial parasites, and in the absence of an exoerythrocytic infection that could elicit $CD8^+$ cytotoxic T lymphocytes, are the most relevant effector cells [1,4,30]. Furthermore, the strategy to identify candidate vaccine antigens with T lymphocytes was used because antigens that elicit a dominant antibody response have generally not induced protective immunity [4,6] and the goal of this study was to identify previously unrecognized, subdominant antigens. This paper describes a new, immunogenic 78 kDa protein from *B. bovis*, designated ACS1 because of the significant identity with other family members of fatty acyl coenzyme A synthetases and the presence of highly conserved signature motifs that play essential roles in catalytic activities, such as ATP binding and fatty acid binding.

B. bovis ACS1 is related to the *P. falciparum* ACS family of proteins [27]. The *B. bovis* ACS1 sequence was used to search the preliminary *B. bovis* genome sequence using TBLASTN and two additional family members, designated ACS2 and ACS3, were identified. The presence of transcripts encoding these proteins indicates that they are all expressed. Alignment of the predicted amino acid sequences revealed 30–44% identity between the three *B. bovis* ACS proteins. This is somewhat less than the identity between the *P. falciparum* ACS1–ACS11 proteins, which ranged from 27 to 79% [27]. *B. bovis* ACS1 has the highest identity (32%) with *P. falciparum* ACS11, while *B. bovis* ACS2 and ACS3 have the highest identity with *P. falciparum* ACS10 (35%). However, the identity of *B. bovis* ACS1–ACS3 to putative long-chain fatty acyl-CoA synthetases of the cattle apicomplexan pathogens *T. parva* and *T. annulata* ranges from 44 to 49% (data not shown).

B. bovis ACS1 was identified by expression library screening with a rabbit antiserum raised against a *B. bovis* fraction that stimulated proliferation of T lymphocytes from several *B. bovis*-immune cattle [21,22]. The present study confirmed that T lymphocytes from one of the original animals (C97) used for the antigen fractionation experiments and T lymphocytes from additional immune cattle 00B16, 00B17, and B49 respond specifically to recombinant ACS1 protein. In contrast, we could not detect ACS1-specific antibody in sera from these and other cattle that had recovered from infection. This result suggests that either ACS1 is not a serologically immunodominant antigen for cattle, or that in cattle, antibody is directed against conformational epitopes.

Repeated attempts to localize the ACS1 protein in *B. bovis*-infected erythrocytes by indirect immunofluorescence using the monospecific mouse sera were unsuccessful, in spite of positive staining with live and fixed merozoites with antiserum specific for MSA-1 (data not shown). In *P. falciparum*, ACS1 protein was not found associated with the merozoite surface membrane, but rather was within vesicles in the erythrocyte cytoplasm [31].

Matesanz et al. [31] suggested that this localization is consistent with fatty acid synthesis required for the development of a tubuvesicular network of membranes that constitutes a nutrient transport system [32]. Additional experiments are needed to determine the localization and temporal expression of ACS proteins in *Babesia* parasites in both erythrocyte and tick stages, as well as the relative levels of protein expressed during the course of infection in cattle.

Protozoa, including *Toxoplasma gondii*, *P. falciparum*, and *Trypanosoma brucei* scavenge fatty acids from host cells for their own metabolic usage [33–35]. To be metabolized, long-chain fatty acids are first activated to coenzyme A derivatives by an enzymatic reaction carried out by ACS proteins. The activated fatty acids then participate in numerous cellular functions in addition to membrane biogenesis, including fat deposition, energy production, protein transport, protein acylation, enzyme activation, and cell signaling [36–39]. Recent studies with *T. brucei* suggest that incorporation of myristic acid into glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins requires conversion to myristoyl-CoA by a parasite ACS [40,41].

The functional activity of ACS proteins has not been characterized in any *Babesia* parasite, and this is the first report that protozoal ACS proteins are immunogenic for a mammalian host. Targeting parasite ACS proteins may be a feasible vaccination strategy against *B. bovis*, if T lymphocyte responses can effectively activate macrophages in the spleen to inhibit parasite growth [42,43]. Despite the ability of *B. bovis* to sequester in microcapillaries of the deep vasculature [3], the importance of the spleen in preventing the development of disease in persistently infected animals is well known, as splenectomy results in recrudescence of parasitemia and disease [44]. It may also be possible to target ACS proteins by chemotherapeutic approaches, which have been shown to be lethal for *P. falciparum* in vitro [45]. In *B. bovis*, GPI-anchored MSA-1 and MSA-2 [46,47], are believed to be important for erythrocyte invasion [7–9,48,49], so interruption of GPI anchor formation by inhibiting important enzymes such as ACS, could have severe consequences for parasite survival. Future studies will need to test these hypotheses.

In summary, this paper reports the identification of novel ACS proteins in *B. bovis* that share sequence identity and conserved motifs present in related protozoal pathogens including *P. falciparum*. ACS1 is immunogenic for cattle that recovered from *B. bovis* infection. The recognition of ACS1 by $CD4^+$ T lymphocytes from genetically diverse cattle immune to *B. bovis* provides a basis for testing the protective property of ACS proteins in cattle.

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